Inhibition of Neutrophil Elastase Suppresses the Development of Skin Tumors in Hairless Mice

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In this study we investigated whether a reduction in neutrophil elastase activity in mice would alter the development of ultraviolet B or chemically induced skin tumors. A mutant strain of neutrophil elastase-deficient mice was developed by crossing beige mice with SKH 1 hairless mice. Ultraviolet irradiation three times per week for 20 wk developed an average of 10 tumors per normal mouse, whereas elastase-deficient hairless mice receiving the same treatment developed only 0.4 tumors per mouse. Benzopyrene administered topically for 20 wk resulted in an average of seven tumors per control mouse, while similar treatment to elastase-deficient hairless mice reduced the tumor count to 0.2 per mouse. Two small molecular weight elastase inhibitors, which were shown to inhibit mouse neutrophil elastase, were administered subcutaneously to normal SKH-1 mice during 16 wk of ultraviolet B exposure. Both inhibitors significantly reduced the incidence of ultraviolet B-induced tumors. When control and elastase-deficient mice were immunized with 2,4,6-trinitrochlorobenzene and oxazolone, both molecules elicited a significant contact hypersensitivity response. Ultraviolet B irradiation prior to immunization at a nonirradiated site completely suppressed the induction of contact hypersensitivity in both the normal and the deficient mice, suggesting that prevention of systemic immunosuppression was not the reason for the resistance to skin tumors observed in the elastase-deficient mice. The results suggest that neutrophil elastase can be an important factor in squamous cell tumor development. *Key words: immunosuppression/TNFR/beige/squamous cell carcinomas. J Invest Dermatol 107:159–163, 1996*

Chronic exposure of the skin to UV radiation (UVR) causes destructive changes in the connective tissue elements of the skin and is the major cause of non-melanoma skin cancer. Early studies with mice (Kripke, 1984; Roberts *et al*, 1989) and recent studies with humans (Yoshikawa and Streilein, 1990a) documented the association of UVR-initiated immune suppression with the development of skin cancer. Direct suppression of cell-mediated immunity by modulation of antigen-presenting cell function and increased secretion of specific cytokines are two means where UVR can influence tumor development (Roberts *et al*, 1989; Ullrich, 1995).

Keratinocytes and macrophages are activated by UVR to secrete tumor necrosis factor α (TNF), a potent cytokine responsible for initiating multiple effects on biologic functions (Kock *et al*, 1990). TNF is implicated as a mediator of inflammation, septic shock, cytotoxicity, and cachexia (Beutler, 1988). In addition, TNF plays an essential role in the induction of immune suppression following UVR (Streilein *et al*, 1994). The results of a recent study using a mutant mouse lacking the TNFR-1 receptor, however, challenges this concept (Kondo *et al*, 1995).

Most inbred strains of mice fail to develop contact hypersensitivity (CHS) following hapten sensitization through UV-irradiated skin. Some strains, however, are UVB-resistant and CHS develops normally after the application of hapten, even though the animal had previously been UV-irradiated (Yoshikawa and Streilein, 1990b). This resistance to UVB is apparently TNF-mediated through the 5′ regulatory region of the *Tnfa* gene (Vincek *et al*, 1993).

In this study, through the use of an elastase-deficient hairless mouse model and specific elastase inhibitors, we show that attenuation of neutrophil elastase activity results in a pronounced diminution in the severity of UVB-induced skin tumors. Mechanisms other than resistance to UVR-induced systemic immunosuppression apparently account for the abatement of skin tumors in the elastase-deficient mice.

MATERIALS AND METHODS

Animals Neutrophil elastase-deficient hairless mice were produced by crossing beige C57BL/6J-bg/bg mice (Jackson Laboratory, Bar Harbor, ME) with SKH-1 hairless mice (Charles River Laboratories, Wilmington, MA) as described previously (Starcher and Conrad, 1995). Beige mice carry a mutation on chromosome 13 that results in a selective deficiency of elastase and cathepsin G activity in the azerurophilic granules of neutrophils (Vassalli *et al*, 1978; Takeuchi *et al*, 1986). Female mice from 6 to 10 wk of age were used in all of the experiments. Mice homozygous for both the beige defect and the hairless trait express the elastase deficiency (hhbb). Littermates that were heterozygous for the beige defect and maintained normal levels of neutrophil elastase activity (hhBb) as well as SKH-1 mice were used for controls.

The mice were exposed to UV radiation with a Dermalight 2001 equipped with a UVB filter (Dermalight Systems, Studio City, CA). This source approaches the sun spectrum with 90% ultraviolet A (UVA) and 10%

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Abbreviations: TNCB, 2,4,6-trinitrochlorobenzene; UVR, ultraviolet radiation; CHS, contact hypersensitivity; TNF, tumor necrosis factor α.

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UVB. Three times per week, groups of five mice were exposed for 30 s with the lamp positioned 8 inches above the mice to provide 0.09 J/cm^2 of irradiation. Minimal erthyema with no burning or scarring was evident with this dose. Tumors began to appear approximately 4 mo after UVB irradiation and became severe by 6 mo. Tumors were counted, and representative tumors were characterized by histology.

Contact Hypersensitivity For the contact hypersensitivity studies, the backs of the mice were exposed to 1 J/cm^2 UVB irradiation with a Westinghouse FS 40 sunlamp. Five days later, groups of irradiated and nonirradiated controls were immunized by painting 30 μl of a 5% solution of either oxazolone or 2,4,6-trinitrochlorobenzene (TNCB) in acetone/corn oil (4:1) on the nonirradiated abdomen. Five days after immunization, all groups were challenged with 5 μl of 1% oxazolone or TNCB on each side of the ears. As a measure of CHS response, two independent investigators assessed ear swelling 24 h later using a micrometer (Mitutoyo, Tokyo, Japan).

Chemical Tumor Induction To elicit chemically induced skin tumors, benzopyrene (20 nmol/100 μl in acetone) was applied three times per week over a period of 5 months to the backs of normal hairless and elastase-deficient hairless mice essentially as described by Moon *et al* (1992).

Mouse Pancreatic Elastase Mouse pancreatic elastase was purified from mouse pancreas removed from mice used in these and other experiments. The pancreas were suspended in 0.02 M Tris buffer, pH 8.8, with 0.05 M $CaCl_2$ and containing 250 μg of trypsin and incubated at 37°C for 4 h to activate the elastase. The crude elastase preparation was dialyzed against 0.05 M sodium acetate buffer, pH 5.9, centrifuged, and fractionated on a carboxymethylcellulose column with a linear gradient of 0.05 M sodium acetate buffer, pH 5.9, and 0.5 M in NaCl. Fractions containing elastase activity were pooled, dialyzed against the 0.05 M acetate buffer, and fractionated on a fast-protein liquid chromatography Mono S 10/10 column with a gradient of 0.05 M sodium acetate buffer, pH 5.9, 0.25 M in NaCl, reaching 100% of the second buffer in 244 min. Active fractions were pooled and refractionated on a Mono S 5/5 column using the same gradient system programed to reach 65% of the second buffer in 35 min. SDS gels showed a single band in the active fractions with a molecular mass of approximately 28 kDa.

Mouse Neutrophil Elastase Mouse neutrophil elastase was partially purified from isolated peritoneal neutrophils as described previously (Starcher and James, 1991).

Elastase Inhibition Small molecular weight elastase inhibitors were obtained from ZENICA (ICI-800355) and Sterling Winthrop (Win 63,759-2). Kinetic studies to determine the IC_{50} were performed using human neutrophil elastase and porcine pancreatic elastase (Elastin Products, Pacific, MO) and our purified mouse pancreatic elastase and partially purified mouse neutrophil elastase. The inhibitors were dissolved in dimethyl sulfoxide and added in concentrations ranging from 0.25 to 2.0 μM to 10 μg of elastase in 0.02 M Tris buffer, pH 8.0. Following 5-min preincubation, 30 μl of 1 μM succinyl-alanyl-alanyl-alanine-*p*-nitroanalide (Elastin Products) was added and the mixture incubated at room temperature.

Inhibitor Blood Levels Circulating inhibitor levels were measured in the serum of mice at various times after intraperitoneal administration of 50 μl of a solution containing 12.5 mg of inhibitor dissolved in 2 ml of dimethyl sulfoxide and brought to 5 ml with sterile water. Acetonitrile (300 μl) was added to 200 μl of serum in a microcentrifuge tube, and the tube was vortexed and centrifuged. The supernatant was removed and evaporated with a vacuum centrifuge (Savant Instruments, Farmingdale, NY). The residue was redissolved in 120 μl of 0.02 M Tris buffer, pH 8.0, containing 0.1% serum albumin and 100 μl was added to 25 μl of 0.02 M Tris buffer containing 250 ng of porcine pancreatic elastase. After incubation for 10 min, 30 μl of 1 μM succinyl-alanyl-alanyl-alanyl-alanine-*p*-nitroanalide was added and the reaction measured at 405 O.D. after 7 min at room temperature.

Elastase Inhibition *In Vivo* One hundred and twenty-five micrograms of each inhibitor was dissolved in dimethyl sulfoxide and water as described above and injected subcutaneously into the back of the neck immediately prior to UVB administration, 4 h later, and again after 24 h. This cycle was repeated 3 times per week for 4 mo. For an additional month the mice were given the inhibitor without UVB. UVB-irradiated control mice were administered the vehicle without the added inhibitor. Nonirradiated controls were not given the subcutaneous injection. At the end of the study the tumors were counted, and representative tumors were excised, formalin-fixed, and stained with hematoxylin and eosin for histology.

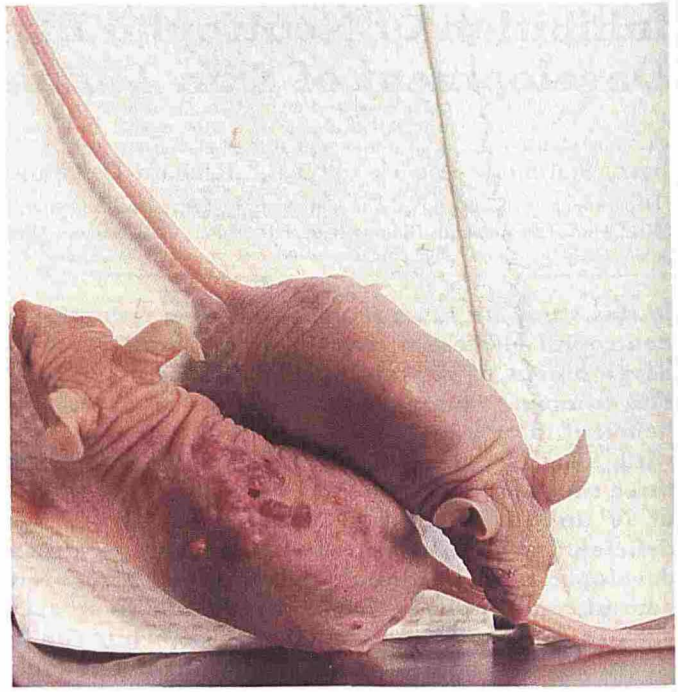

Figure 1. Tumor development on mice exposed to 5.4 J/cm^2 UVB over a period of 5 mo. The control (SKH-1) mouse is on the left, and the elastase-deficient (hhbb) mouse is on the right.

Statistics Data on tumors are presented as total tumors per group of mice. Comparisons between two groups were nonparametric with the Mann-Whitney test using InStat Mac (GraphPad, San Diego, CA). Probability values less than 0.01 were considered significant.

RESULTS

Hairless Beige Mice Are Resistant to UVB-Induced Tumors

Repeated exposure of UVB irradiation to the hairless mice used in these experiments resulted in degenerative changes to the skin consistent with prior observations (Starcher and Conrad, 1995). Inflammatory cells including mast cells, neutrophils, and macrophages accumulated in the dermal layer of the skin and remained in high concentrations throughout the experimental period. The extent of inflammatory cell invasion qualitatively appeared to be the same in both the control and elastase-deficient mice. Tumors started developing on the skin surface exposed to the UVB irradiation at about 14–16 wk of exposure. Virtually all of the larger tumors examined were classified histologically as squamous cell carcinomas with invasion of the dermis by epidermal masses and keratinization in the form of horn pearls. Some tumors, between 2 and 3 mm in diameter, were regarded as papillomas, which may be precancerous but at the time were still benign. Representative smaller tumors (<2 mm) that were classified histologically were always found to be papillomas. We did not determine whether these would become malignant if UVB irradiation were continued.

Perhaps the most notable observation was the absence of tumors on the backs of the elastase-deficient mice after a cumulative dose of 5.4 J/cm^2 of UVB over 5 mo of exposure. **Figure 1** shows a comparison between the appearance of the skin of hhbb and hhBb mice after receiving UVB irradiation for 5 mo. Although their skin was thickened and had the appearance of solar damage, there were no tumors on the backs of the elastase-deficient mice. All of the normal littermates had tumors of varying size distributed over the entire skin surface that was exposed to UVB irradiation. **Table I** shows the tumor count in this experiment and another experiment that used SKH-1 hairless for controls as well as heterozygote littermates. The larger, well developed tumors were classified

Table I. Neutrophil Elastase Deficiency Lowers the Incidence and Numbers of UVB-Induced Skin Tumors[a]

	Tumors >2 mm			
	Experiment 1		Experiment 2	
	Number	Incidence	Number	Incidence
Control hhBb	0	0/5	0	0/5
Control hhbb	0	0/5	0	0/5
UVB hhBb	67	5/5	51	5/5
UVB hhbb	0	0/5	4	2/5
UVB SKH-1			42	5/5

[a] Elastase-deficient (hhbb), normal littermates (hhBb), and SKH-1 mice were administered 0.09 J/cm^2 of UVB 3 times per week for 20 wk. Tumor counts represent the total number of tumors in each group of mice, n = 5. Incidence represents the numbers of mice that had tumors. Control mice were not irradiated with UVB. Tumors on the UVB-irradiated hhbb mice were significantly less in number than the UVB-irradiated hhBb and SKH-1 mice ($p < 0.01$).

histologically as squamous cell carcinomas. Smaller tumors (<2 mm) were also present on the UV-irradiated normal mice but were not recorded in this experiment. If UV irradiation was terminated, the tumors continued to develop, and those that were present never receded during the period of time that we observed them. Over a 3-y period, tumors never spontaneously developed in either the elastase-deficient mice or in their normal littermates.

Chemical Tumor Induction A subsequent experiment was conducted to determine whether the elastase-deficient mice were also less susceptible to tumor formation by a chemical carcinogen. The total numbers of tumors resulting from the topical application of benzopyrene on the backs of five hhbb and six hhBb control mice over a 5-mo period are shown in **Table II**. All of the control hhBb mice developed tumors for a total count of 46 tumors, while only one of the elastase-deficient hhbb mice developed a single tumor. The tumors induced by benzopyrene were generally smaller than those resulting from UVB irradiation. A few large tumors were classified histologically as squamous cell carcinomas.

UVB Suppresses CHS in Control and Beige Mice Mice that were immunized by painting either TNCB or oxazolone on the nonirradiated abdomen and subsequently challenged on the ears demonstrated a significant CHS response as measured by ear swelling (*group 1*; **Figs 2,3**). UVB irradiation prior to immunization (*group 2*) completely suppressed the induction of CHS responses in both the SKH-1 and the elastase-deficient mice. No significant ear swelling was observed in either the irradiated and challenged or the nonirradiated, nonimmunized but challenged control groups.

Elastase Inhibitors Block UVB-Induced Tumors To see whether specific neutrophil elastase inhibitors could modulate tumor induction resulting from UVB irradiation, normal mice, heterozygous for the beige trait (hhBb), were given subcutaneous injections of two small molecular weight inhibitors that were originally designed to inhibit human neutrophil elastase. The results of these experiments are summarized in **Table III**. Tumors that developed in this experiment were divided into those 2 mm or larger (usually identified as squamous cell carcinomas) and small papillomas of less than 2 mm. A total of 21 tumors > 2 mm in size developed on the five hhBb mice receiving UVB, while only two of the mice administered ICI 200355 developed tumors, and none of the WIN 63759-2 inhibitor-treated mice developed tumors of this size. There was a total of 41 small tumors (< 2 mm) on the five UVB-irradiated control mice and a total of nine small tumors on only two of the WIN 63759-2 mice and 15 small tumors on three of the ICI 200355 mice. The numbers of tumors were significantly lower in both the ICI 200355– and the WIN 63759-2–treated mice compared to those receiving UVB alone ($p < 0.01$).

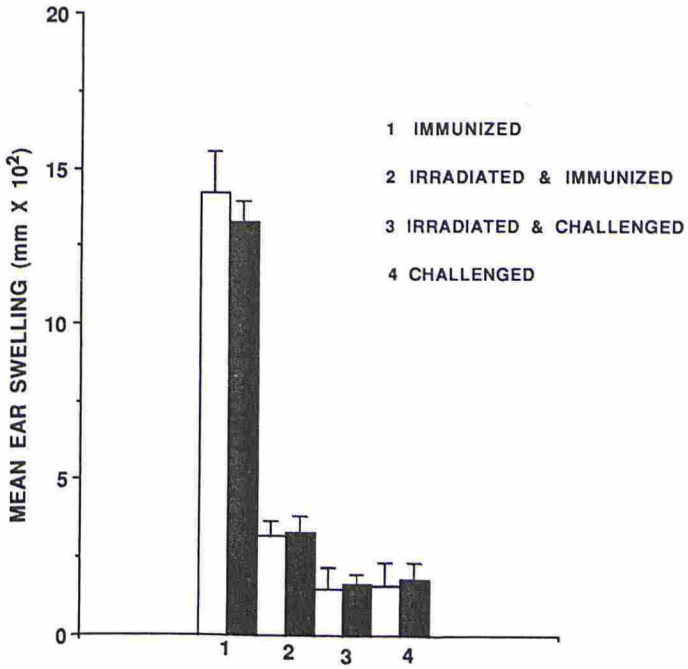

Figure 2. UV-induced suppression of the induction of CHS responses to TNCB. ■, Control (SKH-1) mice represent; □, elastase-deficient (hhbb) mice. Mice were exposed to 1 J/cm^2 UVB irradiation and 5 days later were immunized with TNCB on the nonirradiated abdomen. The mice were challenged 5 days later with TNCB on the ears. Ear swelling was assessed after 24 h. *Error bars*, mean ± SD.

Inhibitor Kinetics The elastase inhibitory capacity of the two small molecular weight inhibitors used in this study, expressed as the molar concentration required for 50% inhibition (IC_{50}), is shown in **Table IV**. A chloromethyl ketone (MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl) was used for comparison as a well recognized and potent general elastase inhibitor. The ICI 200355 inhibitor was very effective in inhibiting all of the sources of elastase that we investigated, exhibiting the same inhibitory capacity toward pancreatic elastases as it did toward neutrophil-derived elastases. The WIN 63759-2 inhibitor demonstrated more specificity than ICI 200355 and was an order of magnitude less effective in inhibiting mouse neutrophil elastase than it was for human neutrophil elastase. This inhibitor showed no inhibitory activity toward mouse pancreatic elastase at levels of more than 10^{-4} M, even though it was fairly effective toward porcine pancreatic elastase.

To see if significant blood levels of these inhibitors could be maintained over reasonable periods of time, 125 μg of each inhibitor was injected intraperitoneally and the blood was assayed for active inhibitor for up to 6 h. Maximum levels of both inhibitors (500–700 ng/ml) were attained 2 h after injection and returned to near baseline levels by 4 h.

Table II. Elastase Deficiency Lowers the Incidence and Numbers of Benzopyrene-Induced Skin Tumors[a]

	Skin Tumors	
	Number	Incidence
Control hhBb	0	0/5
Control hhbb	0	0/5
Benzopyrene hhBb	46	6/6
Benzopyrene hhbb	1	1/5

[a] Elastase-deficient (hhbb) and normal littermates (hhBb) were administered 50 μl of benzopyrene on the skin 3 times per week for 20 wk. Control mice did not receive benzopyrene. Tumor counts represent the total number of tumors including those less than 2 mm in size. Tumors on the benzopyrene-treated hhbb mice were significantly less in number than the benzopyrene-treated hhBb mice ($p < 0.01$).

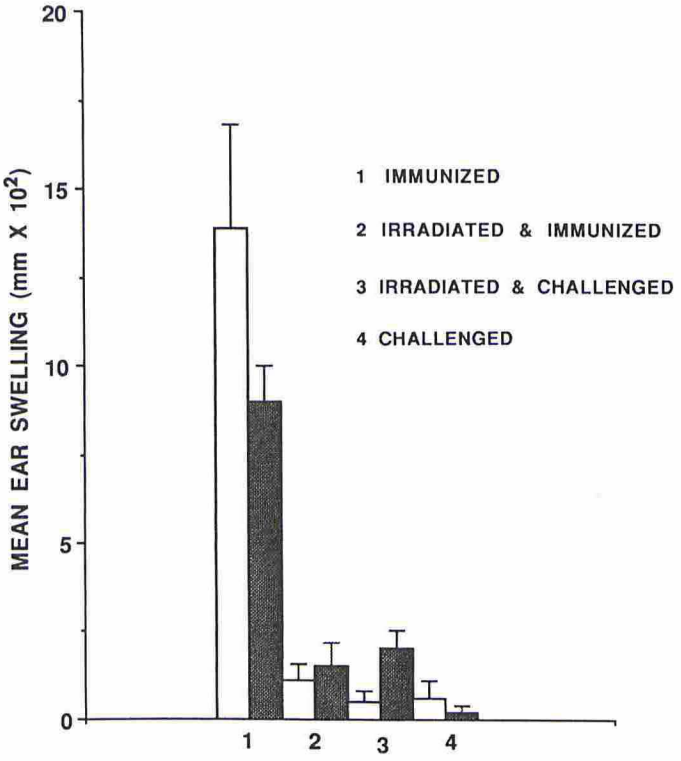

Figure 3. UV-induced suppression of the induction of CHS responses to oxazolone. ■, Control (SKH-1) mice represent; □, elastase-deficient (hhbb) mice. Mice were exposed to 1 J/cm^2 UVB irradiation and 5 days later were immunized with oxazolone on the nonirradiated abdomen. The mice were challenged 5 days later with oxazolone on the ears. Ear swelling was assessed after 24 h. *Error bars*, mean ± SD.

DISCUSSION

Exposure of hairless mice to UVB irradiation over a 4- to 5-mo period produces numerous tumors along the exposed skin surface. This has been a common finding in all experiments of this design (Hsu *et al*, 1975; Kripke, 1977; Forbes *et al*, 1981; DeGruijl *et al*, 1983; Bissett *et al*, 1990; Nishigori *et al*, 1992). In earlier studies on solar elastosis (Starcher and Conrad, 1995), we observed that a hairless-beige mouse cross did not appear to develop skin tumors in response to repeated exposure to UVB. The present studies show that creating a neutrophil elastase deficiency, either genetically or with the use of elastase inhibitors, represses UVB-induced tumor induction. Elastase-deficient mice were also resistant to chemically induced tumors.

One possible explanation was that the elastase-deficient mice were refractory to immunosuppression induced by UVB. Our experiments, however, indicate that hhHb control mice as well as the hhbb mice can be systemically suppressed by UVB for the induction of CHS. This suggests that an inability to express UVB-suppressed immune reactivity is not the reason the elastase-deficient mice were protected against UVB-induced skin tumors. Resistance to the local immunosuppressive effects of UVB radiation, however, as described by Elmets *et al* (1983), cannot be excluded.

Table III. Elastase Inhibitors Repress the Induction of UVB-Induced Tumors

	Skin Tumors[a]			
	>2 mm	Incidence	<2 mm	Incidence
Control	0	0/5	0	0/5
UVB	21	5/5	41	5/5
UVB+Win 63759-2	0	0/5	9	2/5
UVB+ICI 200355	5	2/5	15	3/5

[a] Tumors were divided into larger tumors (>2 mm in diameter), which were usually classified as squamous cell carcinomas, and smaller (<2 mm) tumors, which were diagnosed as papillomas. Tumor counts represent the total number of tumors in each group of five mice per group. Tumors >2 mm and <2 mm on the Win 63759-2 and ICI 200355 inhibitor-treated mice were significantly less in number than the UVB-irradiated control mice ($p < 0.01$).

Table IV. Relative Inhibitory Capacity of WIN 63759-2 and ICI 200355 on Mouse Neutrophil Elastase Activity

	$IC_{50} \times 10^6$			
	Enzyme			
Inhibitor	MNE[a]	MPE	PPE	HNE
WIN 63759-2	3.50	NONE	1.40	0.40
ICI 200355	0.34	0.25	0.25	0.33
CMK	0.33	0.60	0.41	0.61

[a] Abbreviations: MNE, mouse neutrophil elastase; MPE, mouse pancreatic elastase; PPE, porcine pancreatic elastase; HNE, human neutrophil elastase.

The elastase inhibitors used for this study were originally designed for inhibition of human neutrophil elastase. We show in these experiments that mouse neutrophil elastase was also inhibited by both of these molecules and that significant circulating levels of these inhibitors could be maintained over several hours after a single injection. It is doubtful, however, that the subcutaneous injection regimen followed in our UV study maintained sufficiently high blood levels of either inhibitor to protect completely against elastase activity over the prolonged period with elevated skin neutrophils.

Could elastase directly influence the degree to which tumors develop as a result of UVB irradiation? An increase in inflammatory cells in the skin of all the mice following UVB irradiation was a consistent finding throughout these studies. The influx of these cells was evident 12–24 h after the first dose, and the cells remained elevated throughout the the period of UVB exposure. One of the consequences of this influx of inflammatory cells could be the uncontrolled release of proteolytic enzymes. Of these enzymes, neutrophil elastase is of particular interest. Studies by Porteu *et al* (1991) have shown that elastase is capable of removing an active fragment of the TNFR-2 receptors from the cell surface, presumably rendering the cell much less responsive to TNF signaling. Elevated levels of shed TNF receptors will also dramatically reduce the effective biologic activity of circulating TNF (VanZee *et al*, 1992). It is notable that TNFR-1 is the primary receptor responsible for initiating cytotoxicity and is apparently not cleaved by elastase. Removal of TNFR-2 by elastase, however, has been shown to significantly lower the affinity of TNF for TNFR-1 (Tartaglia *et al*, 1993). Other possible roles for elastase could include direct stimulation of tumor growth or of local proliferation of tumor foci. Recent studies by O'Reilly *et al* (1994) have suggested a role for elastase in generating the anti-angiogenesis factor angiostatin, which has a regulatory role in tumor metastasis.

The results of this study demonstrate that a reduction in neutrophil elastase activity can dramatically reduce the incidence of UVB or chemically induced skin tumors in mice. Resistance to UVR-induced systemic immunosuppression apparently does not account for this protective effect. The mechanism of the potential role of neutrophil elastase in squamous cell carcinoma development is unknown.

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